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Kininogenase activity in plasma membranes and cell organelles from rabbit kidney cortex: Subcellular localization of renal kallikrein by free-flow electrophoresis and density-gradient fractionation

HANS G. HEIDRICH and REINHARD GEIGER

Max-Planck-Institut für Biochemie, Martinsried, and the Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik der Universität München, München, Germany

Kininogenase activity in plasma membranes and cell organelles from rabbit kidney cortex: Subcellular localization of renal kallikrein by free-flow electrophoresis and density-gradient fractionation. Subcellular fractions were prepared from a rabbit kidney cortex homogenate by density gradient and free-flow electrophoresis techniques. After enzymatic and morphologic characterization, we determined the kininogenase activity in the different fractions. This activity was present in those plasma membranes that also contained high specific activities of Na-K-ATPase and in lysosomal-like particles. No activity was found in the lumen, that is, the microvillous part of the proximal tubule cell. The kallikrein-like nature of this kininogenase activity was established by several methods.

Activité kininogènasique dans les membranes plasmatiques et les organelles cellulaire de cortex de rein de lapin: Localisation subcellulaire de la kallikréine par d'électrophorèse en flux libre et de fractionnement par gradient de centrifugation. Les fractions subcellulaires ont été préparées à partir de cortex de rein de lapin utilisant les techniques de gradient de centrifugation et d'electrophorèse en fiux libre. L'activité kininogènasique à été déterminée dans les differentes fractions après traitement enzymatique et characterisation morphologique. L'activité à été trouvée dans les fractions plasmatiques contenant également de hautes activités specifiques Na-K-ATPasiques et dans les particles "lysosome-like." Aucune activité n'a été retrouvée dans les zones luminales, i.e., les microvillosités des cellules tubulaires proximales. La nature "kallikrein-like" de cette activité kininogènasique à été établie par differentes methodes.

In 1960 Werle and Vogel found kallikrein activity (EC 3.4.21.8) in rat kidney homogenates [1], and since then several attempts have been made to locate this enzyme in the kidney [2]. The precise location is as yet unclear. The enzyme has been found mainly in the cortical part of the kidney either in glomeruli [3, 4] or in the distal part of the nephron [5–8]. The subcellular localization has also not been unequivocally shown until now. Kallikrein activity has been reported to be present in the microsomal fraction [9], in the endoplasmic reticulum [10, 11], in the plasma membrane [10–13], and in lysosomes [14, 15].

Here we have used several techniques of cell fractionation, such as differential pelleting, density gradient centrifugation and free-flow electrophoresis [16], to unequivocally separate subcellular components from rabbit kidney cortex homogenates. After enzymatically and morphologically characterizing the organelle and membrane fractions, we found very high kininogenase activities in lysosomes or lysosome-like particles and a somewhat lower activity in plasma membranes containing Na-K-ATPase (nonmicrovillous plasma membrane). We also tested specificity of this kallikrein activity.

Methods

Preparation of subcellular fractions. The isolation medium consisted of 10 mM triethanolamine, 10 mM acetic acid, 0.25 mM EDTA, 355 mM sucrose (pH, 7.4; with 2 N sodium hydroxide; osmolarity, 415 mOsm; electrical conductivity, 5.2×10^2 μsiemens).

The kidneys of white/black New Zealand rabbits were perfused with 70 ml of isolation medium, and then 20 to 25 g of cortical tissue was processed according to the isolation scheme (Fig. 1).

For the isolation of lysosomes and lysosomal-like

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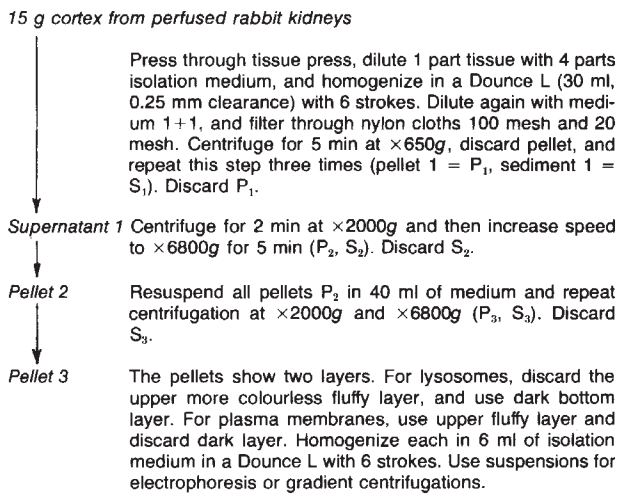


Fig. 1. *Isolation scheme for preparing the starting fraction from a rabbit kidney homogenate for the free-flow electrophoresis and gradient experiments.*

particles by free-flow electrophoresis, the 6 ml of suspension (P3 in Fig. 1) were spun for 20 min at ×100*g* prior to electrophoresis in order to remove aggregates, and the resulting pellet was discarded. Fifteen grams of kidney cortex resulted in 16 mg of total protein, which was injected into a FF-V apparatus (Bender & Hobein, München) according to the method of Hannig and Heidrich [16]. The sample injection was above fraction 65. The conditions of the run were 150 mA, 140 ± 10 V/cm, buffer flow of 2.0 ml/fraction/hr. The fractions were tested for total protein and enzyme activity and then pooled. Lysosomal protein (1.9 mg) was obtained from the 16 mg of protein injected.

Membranes were prepared from the isolated and purified lysosomal organelles by osmotic shock. The particles were resuspended in 10 ml of distilled water, homogenized, after 30 min in ice homogenized again, and then spun down for 20 min at ×100,000*g*. The supernatant was organelle matrix; the pellet was membrane.

Lysosomes and lysosomal-like particles were also isolated by density-gradient centrifugation. The 6 ml of the dark pellet suspension (P3 in Fig. 1) were layered on top of three linear sucrose gradients (57 to 30% wt/wt in isolation medium, 30 ml), centrifuged for 90 min at ×63,500*g* in a swing-out rotor, and the gradients fractionated. The fractions were assayed for total protein and enzyme activity.

For the preparation of luminal microvilli and plasma membranes, the upper fluffy layer suspension (P3 in Fig. 1) was layered on top of the described sucrose gradient and spun as above. After centrifugation, the white layer (34.1 to 37.9% sucrose, that is, density of 1.165 to 1.165 g/ml) was diluted with medium 1+1, centrifuged for 15 min at ×78,500*g*, the resulting pellet washed again with medium, and then resuspended in 2 ml isolation medium. From 15 g of cortex, 5 mg of total protein was obtained and subjected to electrophoresis. The conditions were the same as described above except that the buffer flow was 2.4 ml/fraction/hr. The fractions were assayed for total protein and enzyme activity. Plasma membrane (2.2 mg) and 1.9 mg of microvilli protein were obtained from 5 mg of protein injected.

Protein and enzyme determination. Protein was determined quantitatively in an autoanalyzer (Technicon) as previously described [17]. We determined the following: arylsulfatase activity (EC 3.1.6.1.) [18], acid and alkaline phosphatases (EC 3.1.3.2 and EC 3.1.31) with *p*-nitrophenylphosphate as substrate [19], cytochrome-c oxidase (EC 1.9.3.1) [20], glucose-6-phosphatase activity (EC 3.1.3.9) [21], and Na-K-ATPase (EC 3.6.13) [22]. Enzyme activities are expressed per minute per milligram of protein (specific activities).

For the determination of kininogenase activity (EC 3.4.21.8), kinins were liberated from high molecular weight kininogen as substrate [23]. Kinin activity was tested with the rat uterus assay [3]. Prior to the liberation of the kinins, 0.1 ml of incubation medium (0.15 M Tris and hydrochloric acid buffer [pH, 8.6] containing 4 mM EDTA and 10 mM 8-hydroxyquinoline) and 0.1 ml of substrate (1 mg of kininogen in 0.1 ml of incubation buffer) were preincubated for 30 min at 37° C. The enzyme reaction was started by addition of 0.05 ml of the suspension to be tested (the fractions from the electrophoresis or the gradients were sedimented and the pellets resuspended in 0.05 ml incubation medium). The kallikrein action was stopped after 2 hours by adding 0.05 ml of Trasylol solution (100,000 KIU per 5 ml from Bayer AG, Leverkusen), followed by heating the samples to 100° C for 10 min. Then the kinin activity in the samples was determined using the rat uterus assay [3]. To measure the inhibition of kallikrein-induced liberation of kinins, we sedimented the fractions from the electrophoresis runs, and we resuspended the pellets in 0.1 ml of incubation buffer and halved them. One half was used for kallikrein activity measurement as described above. The other was preincubated for 10 min at 37° C after the addition of 0.05 ml of Trasylol solution (100,000 KIU per 5 ml), and then kininogen was added. The reaction was stopped by addition of Trasylol and boiling as described above. Kinin activity was test-

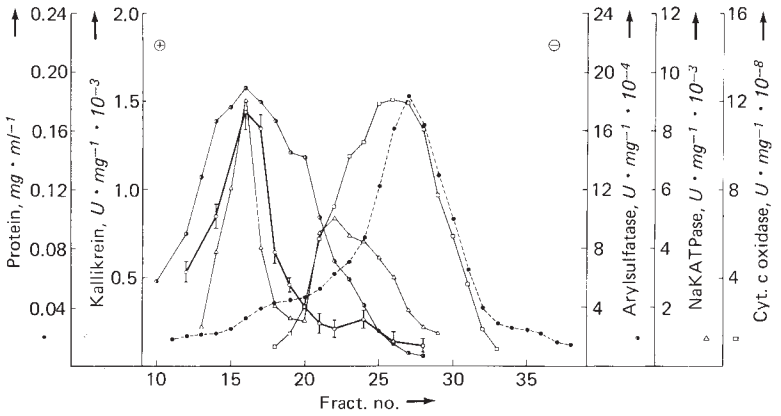


Fig. 2. *Separation profile of a free-flow electrophoresis run for isolating lysosomal organelles.* Enzyme activities are expressed per minute per milligram of protein (specific activities). ●—● protein, ○—○ kininogenase, ◑—◑ arylsulfatase, △—△ Na-K-ATPase, □—□ cytochrome-c oxidase (mean values of eight experiments; SD values are mentioned in the text).

ed in these samples. Parallel samples of lysosomes and plasma membranes were resuspended in sterile 0.9% sodium chloride solution and incubated for 10 min with or without Trasylol. The suspensions were then injected i.v. into a rabbit anesthetized with sodium pentobarbital (Nembutal®). The blood pressure was recorded during the experiment.

Electron microscopy. The fractions were centrifuged at ×37,000*g* for 10 min, the pellets fixed with glutaraldehyde for 1 hr (2% in isolation medium), then in osmiumtetroxide for 60 min, dehydrated, and embedded in Epon 812 or Spurr resin. Thin sections were stained with uranyl acetate and lead citrate and examined in a microscope (JEOL 100 B; 80 kV).

Results

Kininogenase activity in lysosome-like particles. The resuspended dark bottom layer from the × 6,800*g* sediment (P3 in Fig. 1) was already 5.7 times enriched in arylsulfatase activity; that is, in lysosomal organelles and 5.2 times in kininogenase activity.

When this suspension was subjected to electrophoresis in a free-flow system, a distribution was obtained (Fig. 2) similar to that described for the heavy mitochondrial fraction of rat liver [24, 25]. The majority of the material consisted of mitochondria (fractions 20 to 32 in Fig. 2) as demonstrated by the high cytochrome-c activity and morphology (not shown here). Arylsulfatase activity, however, which is representative for lysosomes, was clearly separated (fractions 10 to 23 in Fig. 2) from the mitochondrial activity. Its specific activity was highly reproducible in the peak fraction from experiment to experiment (1.6×10^{-3} U/mg of protein ± [SD] 0.055, $N = 8$). These fractions also contained acid phosphatase activity. The morphology of the particles in these fractions (Fig. 3) demonstrated a population of lysosomes or lysosomal-like particles that was heterogeneous but did not contain any other cell organelles or membranes. As can be seen from Fig. 2, high kininogenase activity (1.5×10^{-3} U/mg of protein ± [SD] 0.35, $N = 8$) was found in the electrophoresis fractions containing the lysosomal activity. This specific activity, however, was not nearly as reproducible in the different experiments as was that of arylsulfatase. It should also be pointed out that within the lysosomal fraction an unexpected peak of Na-K-ATPase was determined, the specific activity of which was highly reproducible (9×10^{-3} U/mg of protein ± [SD] 0.15, $N = 8$). A second lower peak of ATPase activity (fractions 21 to 26 in Fig. 2) was due to the presence of plasma membranes in these fractions (see also Fig. 5 and text below).

To test whether the lysosomal fraction that definitely contained Na-K-ATPase activity was contaminated with plasma membranes or whether this activity was a property of the isolated particles, we also isolated lysosomal particles using a sucrose gradient similar to that described by Maunsbach [26]. Figure 4 shows that arylsulfatase and kininogenase activity coincide in the fractions with a density 1.205 to 1.250 g/ml (fractions 2 to 8 in Fig. 4) as found in the electrophoresis experiments. Na-K-ATPase activity was also present in the lysosomal fraction, and the specific activity in the peak fraction was about the same as it was in the electrophoresis runs. Most of the ATPase activity, however, was found in a less dense region of the gradient where plasma membranes were expected

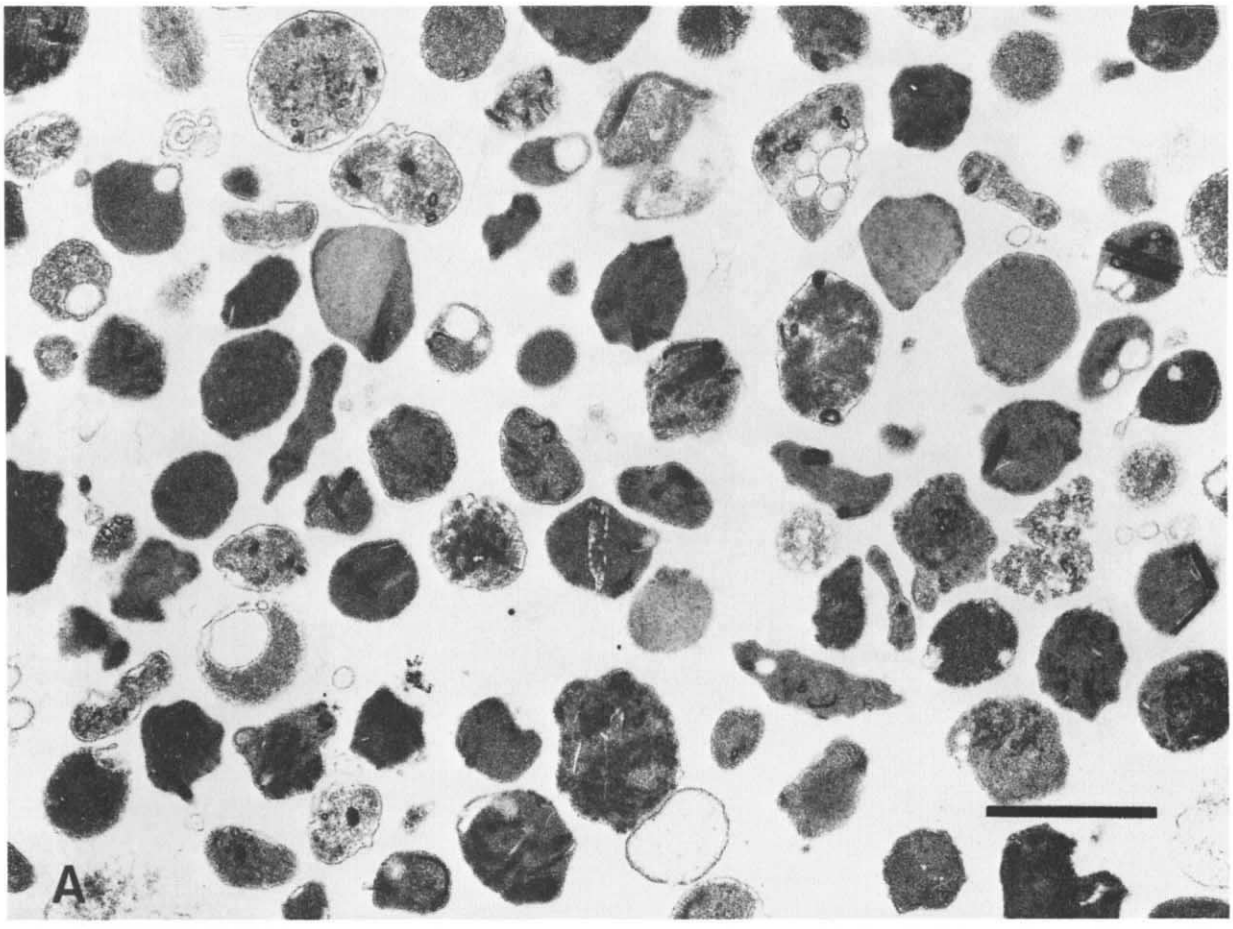
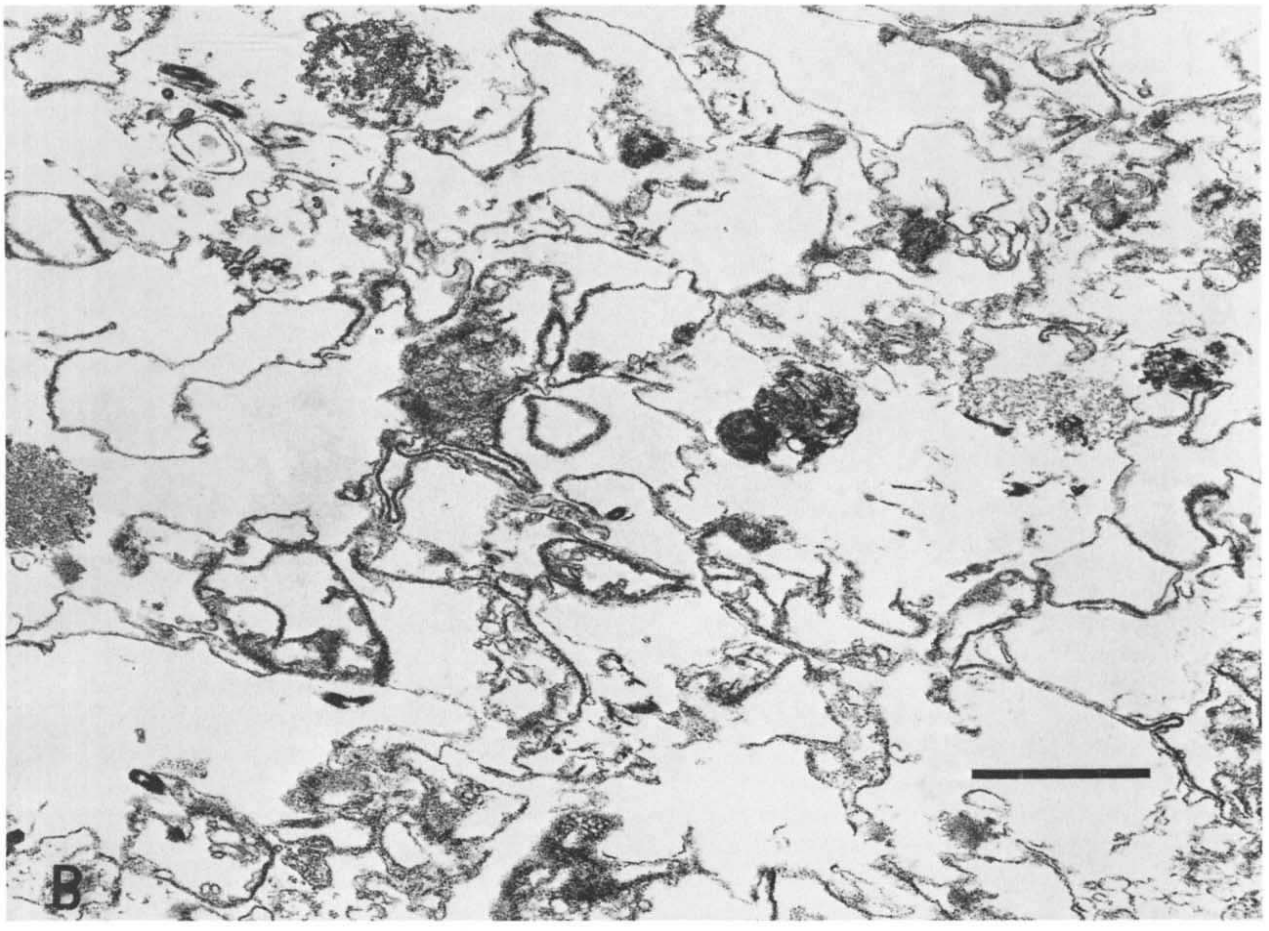


Fig. 3. a *Electron micrograph of lysosomal organelles from rabbit kidney cortex isolated by free-flow electrophoresis (fractions 11 to 19 in Fig. 2).* The particles with dense matrix could be lysosomes or some other type of organelles, for example secretory granules. Bar: 1 μm. **b (next page)** *Membranes obtained after water-lysis of the lysosomal organelles as obtained from free-flow electrophoresis experiments.* Bar: 1 μm.

(density, 1.245 to 1.165 g/ml). Here the specific activity of Na-K-ATPase was 20 × 10^{-3}U/mg of protein ± [SD] 0.15 (N = 3), indicating that these plasma membranes were not very homogeneous (see also Fig. 5 and text below). In this fraction of plasma membranes, a low kininogenase activity was found. The mitochondrial activity in the gradient is not shown in Fig. 4, but it was found in fractions 8 to 12. Endoplasmic reticulum is also not shown here; it was found at a very low level in fractions 9 to 11.

After osmotic water-shock of the isolated lysosomes, most of the kininogenase activity was found not together with the lysosomal matrix enzyme arylsulfatase. This is strong evidence that kallikrein is present in the membrane of the lysosome-like organelles.

Kininogenase activity in plasma membranes. Both the results of the electrophoresis and density gradient experiments and reports of other authors [10–13] indicate that kininogenase activity is present in the plasma membrane. To investigate this further, we made preparation of nonmicrovillous plasma membranes and luminal microvilli using free-flow electrophoresis. Such a separation had previously been carried out successfully for rat kidney [22]. In the present study, the upper fluffy layer from P3 in Fig. 1 was run on a Maunsbach-type of gradient [26] shown in Fig. 4, from which fractions 10 to 14 were collected. This material was then subjected to free-flow electrophoresis. In the electrophoretic separation profile (Fig. 5) of this fraction, Na-K-ATPase as a marker for nonmicrovillous plasma membranes and alkaline phosphatase activity for luminal microvilli are separated clearly from each other (fractions 25 to 34 and 33 to 40, respectively). This distinct separation was already visible in the separation chamber in which two bands could



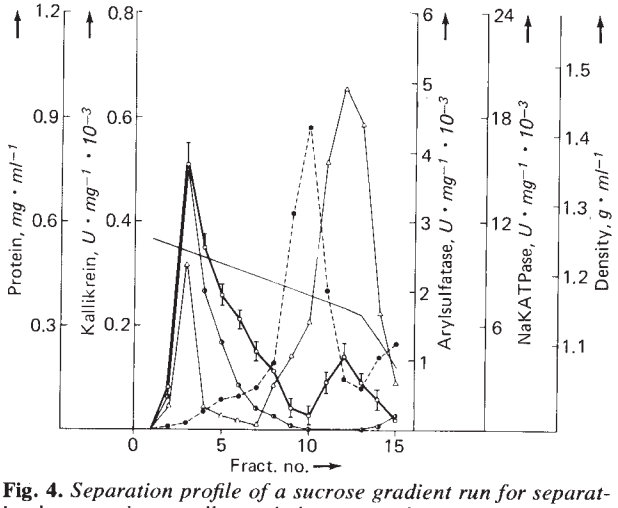


Fig. 4. *Separation profile of a sucrose gradient run for separating lysosomal organelles and plasma membranes.* For the isolation of lysosomal particles, the dark bottom layer of P3 (Fig. 1) was loaded onto the gradient. Then, the yields in fractions 1 to 6 were very high. For the isolation of plasma membranes, the fluffy top layer of P3 (Fig. 1) was loaded on the gradient, and the yield in fractions 10 to 14 was very high. ●—● protein, ○—○ kallikrein, ◑—◑ arylsulfatase, △—△ Na-K-ATPase (mean values of three experiments).

be observed. The specific activity of Na-K-ATPase was very high in the peak fraction (70.0 × 10^{-3} U/mg of protein ± [SD] 0.15, N = 6), indicating that homogeneous plasma membranes had been produced. This is also shown in the electron micrographs (Fig. 6a), although the membranes have a somewhat uncharacteristic vesicular appearance. Kininogenase activity in this fraction was also high (1.25 × 10^{-3} U/mg of protein ± [SD], N = 6) but somewhat lower than it was in the lysosomal particles from the electrophoresis run (1.5 × 10^{-3} U/mg protein ± [SD] 0.35, N = 8). The fractions containing alkaline phosphatase activity (fractions 33 to 40 in Fig. 5) possessed no Na-K-ATPase and no kininogenase activity. From the enzymatic and morphologic characteristics, these particles must be homogeneous microvilli or microvillous vesicles (Fig. 6b).

Discussion

Lysosomal preparation. Kidney lysosomes have been prepared by several authors [26–30] mainly using differential and density-gradient centrifugation

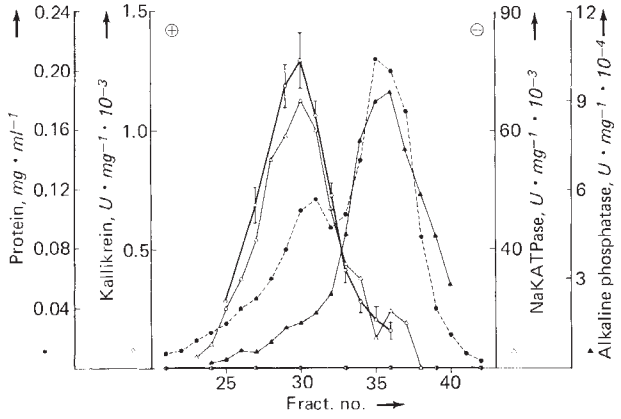


Fig. 5. *Separation profile of a free-flow electrophoresis run from the gradient fractions 10 to 14 (in Fig. 4).* Two separated bands were visible in the separation chamber. ●—● protein, ○—○ kallikrein, △—△ Na-K-ATPase, ▲—▲ alkaline phosphatase (mean value of six experiments; SD values are mentioned in the text).

techniques. These lysosomal fractions have been studied extensively for their enzyme patterns and morphologic and physical properties. There is full agreement that, in principle, the lysosomes from rat kidney resemble those from rat liver, but there are distinct differences [31]. The heterogeneity of kidney lysosomes in situ in size and morphologic appearance is striking, being due probably to the differentiation and specialization of the vacuolar apparatus in the developing tubules. As can be seen from the electron micrographs (Fig. 3), the fraction containing lysosomes and lysosome-like particles is very heterogeneous, even though it does not show other organelles such as mitochondria or membrane fragments. Besides very dense particles, emptier particles and particles containing inclusions similar to those seen in situ can be recognized. Thus, it is unlikely that a selection according to size or density takes place with this technique. Therefore, it must be taken into consideration that this fraction might contain particles that are not secondary lysosomes but, for example, secretory granules with the same electric surface charge (that is, the same membrane surface properties) and the same density. This could explain the appearance of the granules with very dense matrix that can be recognized in the electron micrographs (Fig. 3).

Although this fraction of lysosomes and lysosome-like particles is high in acid phosphatase activity, the most reliable and reproducible enzyme activity was that of arylsulfatase A. Therefore, this enzyme was used routinely as a marker for the lysosomal fraction. It appears not to be bound to the membrane. In this lysosomal fraction, cytochrome-c oxidase activity (as a marker for mitochondria) and NADPH-cytochrome-c reductase activity (as marker for endoplasmic reticulum) were completely absent. There was a reproducible activity of oubain-sensitive Na-K-ATPase present in both the lyso-

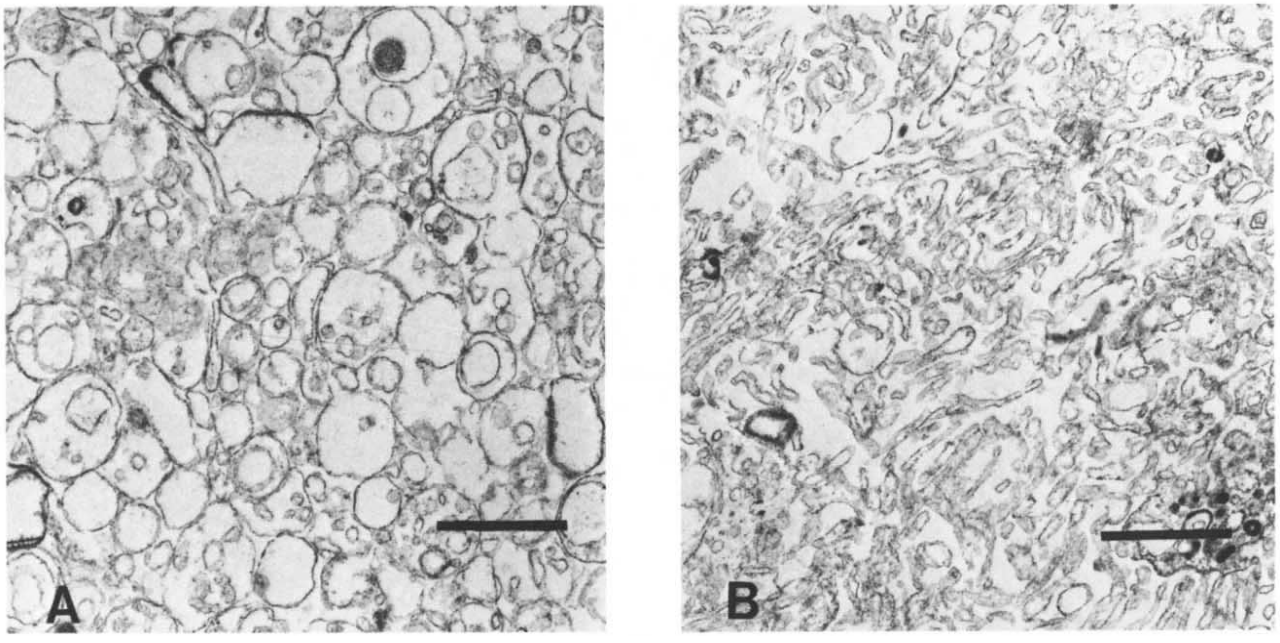


Fig. 6. A *Electron micrograph of the membranes obtained from fractions 25 to 32 of the electrophoresis run (Fig. 5).* Mainly large vesicles can be seen. Bar: 1 μm. **B** *Material from fractions 34–49 of the electrophoresis run (Fig. 5).* Microvilli, microvillous fragments and small vesicles are present. Sometimes the microvilli show a coat. Bar: 1 μm.

somal fraction isolated by free-flow electrophoresis and the lysosomal fraction isolated by density-gradient centrifugation. This activity was low but so reproducible that it cannot stem from contamination with plasma membrane but rather must be a constituent of the membrane of the particles in this fraction. Mego, Farb and Bornes [32] have postulated an energy-dependent (proton) pumping system in the heterolysosomal membrane of mouse kidney, because ATP stimulates the intralysosomal proteolytic activity. Most likely, this ATPase activity is due to the presence of particles produced by fusion of a Golgi vesicle with an endocytotic or a phagocytotic vacuole to form the early developmental stages of secondary lysosomes. Such an explanation for the presence of ATPase activity in lysosomes has been reported by Daems and Persijin [33] and shown by Straus [28] and Wachstein and Besen [34]. According to the "classical" theory, secondary lysosomes do not contain Na-K-ATPase activity.

Plasma membrane preparation. As has been shown earlier in studies from rat kidney [22], free-flow electrophoresis can separate luminal microvilli (having a weak anionic nature) from non-microvillous plasma membranes (having a stronger anionic nature). The same results were obtained in the present study on rabbit. The microvilli also were moved less near to the anode than were the Na-K-ATPase-containing membranes. The distribution of the marker enzymes was distinct: Na-K-ATPase was present only in the more anionic-moving, alkaline phosphatase only in the less anionic-moving membrane fraction. This is proof for a separation of the luminal microvilli and the nonmicrovillous plasma membrane domains. It must be kept in mind, however, that the luminal part of the distal cells contains membrane domains without microvilli. These areas of the membrane could also be isolated together with the basal-lateral Na-K-ATPase-containing membranes from the proximal tubules cells due to similar surface charges and densities. The morphologic interpretation of the results with rabbit was not so obvious as it was with rats. Although in rat the luminal membrane could be recognized as long, well-preserved, single microvilli and the basal membrane as typical sheets of plasma membrane (with junctions), this was not the case in the present study. The fraction with the ATPase activity exhibited plasma membrane vesicles, and the fraction with the alkaline phosphatase activity contained short and long microvilli fragments and very few intact brushborder pieces. These results show a basic difference between rat and rabbit cortex cells: although in the cells from rat a "lifting-off" of the apical from the basal part from the proximal tubule cell is possible, it is not in rabbit. Thus, rat kidney cells are much less stable than are rabbit kidney cortex cells. The microvilli from rabbit proximal tubule cells are not lifted off, but are broken into vesicles [35] by a somewhat stronger homogenization or obtained in the form of intact brushborder fragments.

Localization of kininogenase. The localization experiments for kallikrein should be interpreted bearing the above mentioned considerations in mind. According to the presented results, kininogenase activity is present in high activities in the fraction containing the lysosomes and lysosome-like particles that also contain a low specific activity of Na-K-ATPase activity. It is also present in somewhat lower specific activity in the plasma membrane fraction containing the high specific activity of Na-K-ATPase. The first result is in agreement with some authors [14, 15]; the latter, with others [10–13]. Because in the present work the organelle and membrane fractions have been unequivocally separated from each other by two separate techniques that use different criteria and particle properties for the separation, kininogenase activity must be present in both the fraction containing lysosomes and lysosome-like organelles and in the nonmicrovillous plasma membrane.

To prove whether the tested kininogenase activity in the organelle and membrane-containing fractions was really due to the presence of kallikrein (that is, an enzyme with very restricted substrate specificity, probably to kininogen only) or whether it might have been caused by cleavage of the kininogen substrate by other trypsin-like proteases, we performed the following experiments. *First*, the release of kinins from kininogen was tested with the rat uterus test using the kallikrein activity-containing fractions and found to be positive. *Second*, the release reaction was found to be totally inhibited by Trasylol [36]. *Third*, after the injection of the lysosomal and the plasma membrane extracts into a rabbit, the blood pressure of the animal was immediately decreased by 20 to 30 mm Hg. This decrease was not observed when samples incubated with Trasylol prior to injection were applied to the animals.

Liberation of kinins from kininogen and inhibition of the kininogenase-induced kinin liberation is not a strong proof that the activity is due to a "true" kallikrein, but is at least an indication for the

presence of a trypsin-like enzyme. The lowering of rabbit blood pressure, however, in spite of the presence of a high trypsin-inhibitory capacity in the serum of the animal, strongly supports the assumption that the kinin-liberating activity found in the subcellular fractions is due to a "true" kallikrein. Unequivocal proof that kallikrein activity is present, can only be obtained by, for example, chromatographic isolation of kallikrein from the particles. The quantities obtained in these experiments, however, were insufficient for such a test.

Conclusions. It has been proven that kallikrein-like activity in the kidney cortex is present in subcellular fractions that also contain Na-K-ATPase activity and in organelles that, according to their enzyme markers, have to be classified as lysosomes or lysosome-like particles. These data, together with other reports, can be used to speculate on the origin of urine kallikrein and the transport of kallikrein within or through the cells of the nephron. This is only possible, however, when the exact cell type of the nephron, in which kallikrein is present, is known. There is evidence that kallikreins are synthesized in the kidney [37]. There is also evidence that cells in the distal part of the nephron contain kininogenase activity [5-8] and that kallikrein enters urine at this level. Some of this evidence derives from immunofluorescence experiments. The experiments described here are not contradictory with these results because part of the isolated Na-K-ATPase-containing plasma membranes (with kallikrein activity) could stem from distal cells. Because it is possible now to separate cells from the kidney cortex into distinct populations [38] and because their biochemical characterization is under way [39], clear answers on the localization of kallikrein in the different segments of the nephron can be expected in the future. A necessary precondition for finding the kallikrein active cells, however, is a specific kallikrein assay that is more sensitive than the used bioassay.

Acknowledgments

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Reprint requests to Dr. Hans G. Heidrich, Max-Planck-Institut für Biochemie, D-8033 Martinsried, Germany

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